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# Anti-Folate Receptor alpha-directed Antibody Therapies Restrict the Growth of Triple Negative Breast Cancer

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## Running title:

Anti-FR $\alpha$  antibody against triple negative breast cancers

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## Disclosure of Potential Conflicts of Interest

S.N. Karagiannis and J.F. Spicer are founders and shareholders of IGEN Therapeutics Ltd. F.O. Nestle is an employee of Sanofi US. All other authors have declared that no conflict of interest exists.

## Translational Relevance

Triple negative breast cancer (TNBC) represents a molecularly and clinically diverse disease with cytotoxic chemotherapy the only systemic treatment modality, and no targeted agents approved in adjuvant, neoadjuvant, or metastatic settings. We demonstrate that a significant population of aggressive high-grade TNBCs overexpress the cell surface tumor-associated antigen Folate Receptor alpha (FR $\alpha$ ) and molecules involved in folate metabolism. Importantly, FR $\alpha$  is expressed in post-neoadjuvant chemotherapy residual disease, associated with worse clinical outcomes, and participates in cancer cell signaling and growth. We show that FR $\alpha$  may present a promising target for therapeutic strategies such as antibody-drug conjugates, or antibody immunotherapy that primes an Fc-mediated anti-tumor immune response *in vitro* and *in vivo* in the human patient breast cancer and the patient immune context. Engineering antibodies targeting FR $\alpha$ -expressing breast cancers may provide new strategies to treat patients with poor prognosis who do not adequately benefit from currently-available targeted, and immunology therapies.

## Abstract

**Purpose:** Highly-aggressive triple negative breast cancers (TNBCs) lack validated therapeutic targets and have high risk of metastatic disease. Folate Receptor alpha (FR $\alpha$ ) is a central mediator of cell growth regulation that could serve as an important target for cancer therapy.

**Experimental Design:** We evaluated FR $\alpha$  expression in breast cancers by genomic (N = 3414) and immunohistochemical (N = 323) analyses and its association with clinical parameters and outcomes. We measured the functional contributions of FR $\alpha$  in TNBC biology by RNA interference and the anti-tumor functions of an antibody recognizing FR $\alpha$  (MOv18-IgG1), *in vitro* and in human TNBC xenograft models.

**Results:** FR $\alpha$  is overexpressed in significant proportions of aggressive basal like/TNBC tumors, and in post-neoadjuvant chemotherapy-residual disease associated with a high risk of relapse. Expression is associated with worse overall survival. TNBCs show dysregulated expression of thymidylate synthase, folate hydrolase 1 and methylenetetrahydrofolate reductase, involved in folate metabolism. RNA interference to deplete FR $\alpha$  decreased Src and ERK signaling and resulted in reduction of cell growth. An anti-FR $\alpha$  antibody (MOv18-IgG1) conjugated with a Src inhibitor significantly restricted TNBC xenograft growth. Moreover, MOv18-IgG1 triggered immune-dependent cancer cell death *in vitro* by human volunteer and breast cancer patient immune cells, and significantly restricted orthotopic and patient-derived xenograft growth.

**Conclusions:** FR $\alpha$  is overexpressed in high-grade TNBC and post-chemotherapy residual tumors. It participates in cancer cell signaling and presents a promising target for therapeutic strategies such as antibody-drug conjugates, or passive immunotherapy priming Fc-mediated anti-tumor immune cell responses.

## Introduction

Triple negative breast cancer (TNBC), defined by lack of oestrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) expression, represents an urgent unmet clinical need for treatment options. This is largely due to its aggressive nature and lack of suitable therapeutic targets. TNBC is a heterogeneous disease at the cellular and molecular levels, with its diverse phenotypes correlating with different drug resistance and clinical outcomes (1). Gene expression profiling and expression signatures have identified five molecularly-distinct types of breast cancers, including ER-positive luminal (luminal A and B), HER2-positive, normal-like and basal-like (BL) subtypes. The majority of BL carcinomas have a high mitotic rate, and are usually triple-negative (2). Different TNBC subgroups also correlate with risk factors, incidence, prognosis and treatment response (3). The Lehman-Pietenpol expression classification crystallizes six further TNBC subtypes with implications for prediction of prognosis and chemotherapy sensitivity (4). Although TNBCs are largely defined by a clinical diagnosis of exclusion based on pathological parameters, together these studies point to the potential for identification of disease-associated markers which may serve to define patient subgroups and lead to personalized targeted therapy.

At present, no targeted treatments are standard of care for TNBC. Antibodies recognizing growth factor receptors such as cetuximab or bevacizumab (5, 6), and small molecule drugs such as dovitinib and cabozantinib (7, 8), have been explored in clinical trials, alone or in combination with chemotherapy. These have shown relatively limited response rates in unselected patient populations (9), most likely due to activation of alternative compensatory pathways and inter-/intra-tumoral heterogeneity in expression and mutational status, which may be responsible for intrinsic and acquired resistance-driving mechanisms (10). Thus, disease management mostly relies on a combination of surgery, radiotherapy and multiple chemotherapeutic agents, often associated with high risk of local and systemic relapse (11).

Folate Receptor alpha (FR $\alpha$ ) and its ligand folate are central mediators of cell growth regulation for the one-carbon metabolic reaction and DNA biosynthesis, repair and methylation (12). Insights into FR $\alpha$  distribution (high expression in tumors and restricted expression in normal tissues), alongside emerging roles in cancer growth and metastasis have led to renewed interest in this as a therapy target (13, 14). Preclinical and clinical anti-tumor activities of FR $\alpha$ -targeted therapies have thus far mostly been examined in the context of lung and ovarian carcinomas. These include monoclonal antibodies

farletuzumab (15) and MOv18-IgG1 (16), antibody-drug conjugate (ADC) Mirvetuximab Soravtansine (17), and small molecule drug vintafolide (18). Encouraging results have recently been reported for the thymidylate synthase inhibitor ONX-0801 in ovarian carcinoma (19). The FR $\alpha$ -targeted hapten immunotherapeutic regimen, Folate Immune, was designed to render tumors more immunogenic; however, a phase II trial in renal carcinoma was terminated due to low patient accrual (NCT00485563). Another phase I trial of a FR $\alpha$ -specific chimeric antigen receptor (CAR)-T cell therapy in ovarian cancer patients showed no reduction in tumor burden (20). Recently, Song *et al.* showed that new generation FR $\alpha$ -specific CAR-T cells significantly inhibited high FR $\alpha$ -expressing TNBC xenograft growth (21). However, monoclonal antibody therapeutics agents targeting FR $\alpha$  are yet untested in TNBC.

In this study, we examined FR $\alpha$  as a target for monoclonal antibody therapy approaches. We ascertained the clinical and biological significance of FR $\alpha$  in TNBC and the largely overlapping basal-like subtype, associations of FR $\alpha$  expression with clinical parameters and outcomes by genomic and immunohistochemical analyses. We employed RNA interference and cell-based functional assays to interrogate how FR $\alpha$  may contribute to breast cancer cell biology. We studied FR $\alpha$  and its downstream folate pathway as therapeutic targets by assessing the potential anti-tumor functions of an antibody recognizing FR $\alpha$  (MOv18-IgG1); as an ADC to inhibit cellular viability *in vitro* and tumor growth *in vivo*; and as immunotherapy to activate human immune cells against TNBC *in vitro*, in orthotopic and patient-derived tumor xenografts (PDTX) *in vivo*, more likely able to recapitulate the complexity and heterogeneity of human disease (22). Our findings define FR $\alpha$  as a promising target for antibody therapies for basal-like breast carcinomas including TNBCs.

## Materials and Methods

### Cell lines

Cell lines were obtained from King's College London (KCL) Breast Cancer Now Unit, except HDQ-P1, purchased from Leibniz Institute DSMZ. Cell lines were authenticated by short tandem repeat profiling. Cells used once tested negative for mycoplasma and used up to 30 passages.

### Gene expression data of human breast cancers

The KCL Guy's Hospital, METABRIC and TCGA Breast cohorts (N=2012) interrogated were previously reported (23, 24, 25). Statistical analyses and respective data plots were generated in R version 3.2.2.

### Tissue microarray (TMA) and immunohistochemistry (IHC)

Primary breast carcinomas from 305 patients with no prior neoadjuvant therapy, and 18 surgical specimens from TNBC post-neoadjuvant chemotherapy (post-NACT) residual cancer burden II/III residual cases were evaluated. Access to pseudo-anonymized samples and clinical data were obtained in accordance with the terms and conditions of National Health Service Research Ethics Committee approved Guy's and St Thomas' Research Tissue and Data Bank (REC No 07/H0804/131). PDTX TMA collection included 26 TNBC tumors obtained by directly implanting patient material orthotopically into NSG mouse mammary fat pads. Staining was performed with FR $\alpha$  IHC Kit (BioCare Medical) following manufacturer's protocol, with additional 30 min anti-FR $\alpha$  incubation (API3005AA). IHC with CD45 (Thermo Fisher Scientific) was detected using the DAKO EnVision System HRP Kit (peroxidase activity visualized with 3,3-diaminobenzidine). Analyses were performed using digital images by NanoZoomer HT Digital Pathology Scanning System (Hamamatsu).

### siRNA- and lentiviral-mediated RNA interference

FR $\alpha$ -targeting siRNA sequences and scrambled duplex were purchased from OriGene. Transient transfection was performed using Lipofectamine2000 (Thermo Fisher Scientific). Viral particles of Tet-pLKO-puro plasmid encoding FR $\alpha$ -specific (5'-GGATGTTTCCTACCTATATAGATTC), and non-targeting sequence (5'-GCGCGATAGCGCTAATAATTT) were generated by transfection into 293T cells. CAL51



were transduced using 1:30 viral dilution of isolated viral supernatant. Successfully transduced clones were selected after 48 hr with 3 µg/ml of puromycin for 5 days, and 1 µg/ml of doxycycline was used to induce FRα knockdown.

### ***In vitro* viability and clonogenic assay**

Cell viabilities were detected by methyl tetrazolium assay (Promega). Optical absorbance was read on FLUOstar Omega spectrophotometer (BMG Labtech) to determine viable cell counts after 96 hr. For clonogenic assays, cells were fixed by methanol, stained with crystal violet solution (Sigma Aldrich). Colonies were measured as a function of mean pixel density per well. Image analysis was performed using ImageJ.

### **Western blot and Human Phospho-Kinase Antibody Array**

Immunoblottings were analyzed with anti-phospho-ERK1/2 (Thr202/Tyr04) (Biolegend) and anti-ERK1/2 (Cell Signalling). Proteome Profiler Human Phospho-Kinase Antibody Array (R&D Systems) were incubated with 470 µg lysate overnight at 4°C. The following day, chemiluminescent detection was done according to manufacturer's protocols. Densitometry analysis was performed using ImageJ.

### **Antibody-drug conjugate production**

MOv18-IgG1 was linked to streptavidin overnight using Lightning-Link Streptavidin Conjugation Kit (Expedeon) according to manufacturer's protocol. A-419259 (Cayman Chemical) was biotinylated using EZ-Link-Sulfo-NHS-Biotin (Thermo Fisher Scientific): 10 mM solution in PBS added to 10 mM EZ-Link-Sulfo-NHS-Biotin in ultrapure water (molar ratio 8:1), incubated at room temperature for 30 min, 133 µl of 1.1 mM solution of biotinylated A-419259 was added per 1 mg of streptavidin-conjugated MOv18-IgG1, followed by 30 min incubation. The ADC was purified by centrifugation using 3K Amicon ultra centrifugal filters six times, then resuspended in PBS for functional experiments.

### **Fluorescence-based tumor cell killing assays**

For live-dead cell cytotoxicity imaging, cancer cells were stained with 5 µM CFSE (Life Technologies). The following day, human peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll Paque PLUS (GE Healthcare) and stained with CellTracker Blue dye (Thermo Fisher Scientific). PBMCs were incubated with cancer cells and MOv18-IgG1



or isotype antibody. Ethidium homodimer-1 (4  $\mu$ M) (Thermo Fisher Scientific) served to label dead cells. Samples were imaged using Eclipse Ti-2 inverted microscope equipped with Nikon DS-Qi2 sCMOS camera and running NIS Elements. Antibody-dependent cell-mediated killing of tumor cells was quantified as previously described (26). Data were acquired using FACSCanto flow cytometer (BD Biosciences).

### ***In vivo* procedures**

Animals were handled in accordance with Institutional Committees on Animal Welfare (The Home Office Animals Scientific Procedures Act, 1986). Antibody-drug conjugate: Six-week-old female CD-1 nude mice were used for orthotopic injection of  $1 \times 10^6$  CAL51 cells (50  $\mu$ l PBS mixed in 50  $\mu$ l Matrigel) (day 1). On day 5, mice received single intravenous injection of 7.5 mg/kg ADC or MOv18-IgG1, or 5 mg/kg A-419259. Tumors were measured with calipers and volumes calculated ( $\pi \times \text{length} \times \text{width}^2 / 6$ ). Experiments were terminated after 28 days when tumor sizes were  $\leq 525 \text{ mm}^3$ . Antibody immunotherapy: Female NSG mice were orthotopically injected with  $1 \times 10^6$  CAL51 cells or  $0.25 \times 10^6$  WHIM02 PDX single cell digests in 50  $\mu$ l PBS:50  $\mu$ l Matrigel (day 1). For CAL51, on days 5 and 19, each mouse received  $12 \times 10^6$  human peripheral blood lymphocytes (PBLs) (following red blood cell lysis of human blood) intravenously and 5 or 10 mg/kg MOv18-IgG1. Subsequent antibody was given once per week. For WHIM02, on days 5 and 18,  $12 \times 10^6$  human PBLs and 10 mg/kg antibody per mouse were given intravenously. Antibody doses were given three times in week one and subsequently twice per week. Experiments were terminated after 33 days for CAL51 and 20 days for WHIM02, and tumor sizes were  $\leq 525 \text{ mm}^3$ . Tumor engraftment of human immune cells was confirmed by IHC staining and flow cytometry (antibody panel: rat anti-mouse CD45-V500; mouse anti-human CD45-PE-Cy7, CD20-APC, CD3-APC-Cy7, CD68-PE (all BD Biosciences), CD14-PE (eBioscience), CD56-PercP Cy5.5 (Cambridge Bioscience).

### **Statistical analyses**

GraphPad Prism was used for statistical analyses. Data were presented as mean  $\pm$  standard error of the mean (SEM). Differences with  $P$ -values  $< 0.05$  were considered statistically significant and all tests were two-sided.

Please see Supplementary Experimental Procedures for more detailed methods.

## Results

### Gene expression pattern of FR $\alpha$ reveal associations with TNBC and basal-like breast cancer

We investigated whether FR $\alpha$  (*FOLR1*) is expressed in TNBC and its majority basal-like subtype by interrogating three transcriptomic datasets: METABRIC (N = 1197) (25), The Cancer Genome Atlas (TCGA) (N = 638) (24) and the KCL TNBC-enriched cohort (N = 177) (23). When tumors were stratified by IHC-defined status, *FOLR1* levels were significantly higher in TNBC compared with non-TNBC (Fig. 1A). *FOLR1* expression was also higher in the basal-like molecular subtype defined by PAM50 classification (Fig 1B). The small changes of DNA copy number in the genome suggest that copy number had an insignificant impact on *FOLR1* expression (Supplementary Fig. 1A). *FOLR1* was expressed in all TNBC subtypes as classified by the Lehman-Pietenpol method (Supplementary Fig. 1B).

Although plasma folate levels have not been clearly associated with breast cancer risk (27), low folate status can lead to hypomethylation, subsequent dysregulation of one-carbon metabolism and DNA instability. This may in turn influence the levels of folate receptors or folate carriers (28). However, the association between breast cancer and folate status in the tumor microenvironment remains undetermined. We investigated three molecules involved in folate metabolism that may be of therapeutic interest in cancer. We found that mRNA levels of methylenetetrahydrofolate reductase (*MTHFR*), a key enzyme in the folate metabolic pathway, are significantly decreased in METABRIC and TCGA cohorts, although not in the KCL dataset (possibly due to enriched TNBC and low non-TNBC patient tumor groups). Furthermore, Thymidylate Synthase (*TYMS*), a folate-dependent enzyme involved in the biosynthesis of thymidine for DNA synthesis and repair (29), was upregulated in all datasets. There was no significant correlation between *FOLR1* and *MTHFR* or *TYMS* expression, suggesting that FR $\alpha$  and folate carriers may be independently-regulated in tumors. Notably, expression of folate hydrolase 1 (*FOLH1*, also known as prostate specific membrane antigen (*PSMA*)), a transmembrane folate hydrolase overexpressed in prostate and breast cancers (30), was higher in TNBC/basal-like subtypes. We found a weak correlation between *FOLH1* with *FOLR1* expression (Pearson's  $r = 0.31$ ,  $p < 0.0005$ ) (Fig. 1C), perhaps suggesting collaborative roles in the tumor microenvironment.

Furthermore, the ten-year overall survival (OS) of patients with high FR $\alpha$  tumor expression was significantly lower than those with medium/low expression (Hazard Ratio (HR) = 1.38,  $P$  = 0.0097) in all breast cancers (N = 1402) (Fig. 1Di). Survival analysis of the TNBC patient subset revealed that despite small cohort size (N = 47), high FR $\alpha$  expression correlated with decreased OS (HR = 3.35,  $P$  = 0.043) (Fig. 1Dii).

Thus, elevated FR $\alpha$  gene expression and dysregulated expression of molecules involved in folate metabolism were detected in basal-like breast carcinomas including TNBCs, and FR $\alpha$  expression was associated with worse patient outcomes.

### **FR $\alpha$ membrane expression by immunohistochemical evaluations**

We next examined FR $\alpha$  expression on a cross-sectional study of breast carcinoma TMA specimens (N = 323, of which 76 were TNBCs) (Fig. 2A). In contrast to a restricted distribution pattern in normal tissues (31), high frequency (>70%) FR $\alpha$  protein expression correlated with high grade disease (Grade I: 0.0%; II: 7.7%; III: 24.7%). Three quarters of the FR $\alpha$ -positive grade III samples displayed a medium (41-70%) or a high (71-100%) percentage of cancer cells with membrane FR $\alpha$  immunostaining. Expression was less frequently-associated with ER-positive (7.5%), HER2 (15.6%) or luminal (6.5%) tumors, relative to ER-negative tumors (30.0%) and TNBC (36.8%) (by IHC classification). FR $\alpha$  expression in TNBCs vary among studies, from 20% (32) to 67% or 80% positivity (33, 34). According to PAM50 molecular classification, we observed FR $\alpha$  expression in 10.7% of HER2-positive and 7.1% of luminal A cancers, while FR $\alpha$  expression was more common in basal-like subtypes (33.3%) (Supplementary Table 1) in concordance with a recent report (32). Furthermore, cell membrane FR $\alpha$  immunostaining, significantly correlated with mRNA expression in the same patient samples ( $P$  < 0.0005) (Fig. 2B). We also found that 13% of samples negative for membrane FR $\alpha$  demonstrated cytoplasmic, non-membrane, FR $\alpha$  staining; these tissues may not be amenable to anti-FR $\alpha$  antibody treatment.

Due to lack of effective targetable oncogenic drivers, treatment for TNBC commonly involves cytotoxic chemotherapy, often given prior to surgery. A subpopulation of chemotherapy-resistant residual tumor cells remaining in breast tissue may be responsible for high metastatic recurrence rates and poor long-term clinical outcomes (35, 36). In a TMA containing eighteen TNBC samples from patients with residual disease post-NACT, we found FR $\alpha$  expression in 61.1% of residual tumors (Fig. 2C), and >75% of positive

samples displayed medium or high percentage of cells with membrane FR $\alpha$  immunostaining.

In summary, more TNBC specimens have FR $\alpha$ -positive immunostaining than other breast cancer subtypes. This is particularly marked in post-NACT residual disease, suggesting that FR $\alpha$  could be therapeutically targeted.

### **FR $\alpha$ expression contributes to breast cancer growth**

To gain insights into FR $\alpha$  functions, we evaluated FR $\alpha$  expression in twenty-two breast cancer cell lines by flow cytometry using the monoclonal antibody MOv18-IgG1 (Fig. 3A, Supplementary Fig. 2A-C). Protein expression correlated with transcriptomic expression (Cancer Cell Line Encyclopedia (CCLE)) (Spearman's rank coefficient,  $r = 0.5784$ ,  $P < 0.01$ , Fig. 3B). Three cell lines (CAL51, T47D and HDQ-P1) showed the highest levels of FR $\alpha$  expression by mRNA and corresponding cell surface expression.

Traditionally FR $\alpha$  has been viewed as an intracellular transporter of soluble folate. However, recent findings indicate that FR $\alpha$  may form macromolecular complexes in which it may contribute to upregulation of oncogenic STAT3/JAK pathways (37) and Lyn signaling (38). We therefore hypothesized that FR $\alpha$  expression may confer a proliferative advantage to high-expressing tumors. Employing RNA interference, we found that FR $\alpha$  expression (mean fluorescence intensity, MFI) in cells treated with FR $\alpha$ -targeting siRNA (siFR $\alpha$ ) was significantly lower than in those treated with non-targeting siRNA (siNT) (Supplementary Fig. 2D). Reduction in FR $\alpha$  was accompanied by reduced cell viability compared with scrambled siRNA-treated cells (Fig. 3C, Supplementary Fig. 2E). FR $\alpha$  knockdown also resulted in reduction of colony formation (Fig. 3C, Supplementary Fig. 2F). Neither viability nor colony formation were affected by FR $\alpha$  knockdown in low FR $\alpha$ -expressing MDA-MB-231 cells.

To further interrogate the contribution of FR $\alpha$  to breast cancer biology, we created a stable, doxycycline-inducible, FR $\alpha$ -knockdown CAL51 cell line. The resulting shRNA-transduced cells had a four-fold lower mean relative FR $\alpha$  expression measured by flow cytometry when compared with cells transduced with non-targeting sequence (shNT). In concordance with siRNA experiments, FR $\alpha$  knockdown led to a modest reduction in proliferation (to  $75.9 \pm 2.2\%$  viability ( $\% \pm \text{SEM}$ ;  $P < 0.0005$ ), and reduced colony formation ability (to  $65.8 \pm 23.1\%$ ,  $P < 0.05$ ). Furthermore, consistent with proposed roles in downstream oncogenic signaling pathways such as STAT3/JAK, we measured a

significant decrease in phosphorylated ERK activity to  $30.4 \pm 7.2\%$  ( $P < 0.0005$ ) with FR $\alpha$  knockdown, suggesting reduction of another proliferative signaling pathway (Fig. 3D, Supplementary Fig. 3A).

Furthermore, we studied the anti-tumor effects of raltitrexed, a highly-selective inhibitor of thymidylate synthase, the key enzyme in folate metabolism (Fig. 1). CAL51 shNT cells (with high FR $\alpha$  expression levels) were more sensitive to raltitrexed compared to FR $\alpha$ -knockdown cells in both normal or folate-free conditions (Fig. 3E).

Together, these data indicate that FR $\alpha$  plays key roles in cell growth and TNBC may be sensitive to therapeutic treatment targeting the folate cycle.

### **FR $\alpha$ -dependent signaling functions in breast cancer cells**

We sought to interrogate the molecular signaling processes regulated by FR $\alpha$  and explore the development of targeted therapeutic options. Signaling pathways were assessed using a human phosphokinase array studied in shNT- and shFR $\alpha$ -transduced CAL51 (Fig. 4A). FR $\alpha$  knockdown significantly decreased the activity of several members of the Src-family non-receptor tyrosine kinase, Lyn, Fyn, Hck and Src, their downstream effector molecule ERK, and of the anti-apoptotic protein CREB. Moreover, FR $\alpha$  silencing was associated with increased activity of the metabolic regulator AMPK $\alpha$ 1 and the cell cycle regulator p53 (S15 and S392), when compared with control shRNA treatment.

Growth inhibition by the broad spectrum Src-family kinase inhibitor A-419259 was observed in cell lines with high FR $\alpha$  expression, but not with MDA-MB-231 which express near background levels of FR $\alpha$  (Fig. 4B). Growth inhibition was also demonstrated in CAL51 using a second Src-family kinase inhibitor AZM475271 (Supplementary Fig. 3C). The A-419259 inhibitor (50nM) also significantly reduced colony formation to  $31.2 \pm 4\%$  ( $P < 0.0005$ ) and ERK activation to  $54.6 \pm 9.2\%$  ( $P < 0.005$ ) (Fig. 4B).

These data suggest that FR $\alpha$  is upstream of multiple Src-family kinases and ERK (Fig. 4C), known to be involved in breast cancer biology, and identify FR $\alpha$  as cell surface molecule associated with signaling and growth, with potential to be targeted in therapeutic strategies for TNBC.

The anti-FR $\alpha$  antibody MOv18-IgG1 exerted a very modest direct inhibition in cell viability under folate-reduced conditions (0.4nM folate) (remaining viability:  $86.3 \pm 2.5\%$ ,  $P < 0.05$  for 10  $\mu$ g/ml;  $87.5 \pm 0.9\%$ ,  $P < 0.005$  for 50  $\mu$ g/ml), when compared with media alone

controls (Supplementary Fig. 3D). This suggested that any direct anti-tumor effects of these agents may be limited to folate-depleted environments, perhaps akin to conditions found in tumors.

MOv18-IgG1 did not engender significant direct inhibition of FR $\alpha$ -dependent signaling under physiological conditions. We therefore developed a FR $\alpha$ -targeting antibody-coupled inhibitor ADC strategy by conjugating the Src-family kinase inhibitor A-419259 to MOv18-IgG1, using the antibody as a vehicle to specifically deliver the inhibitor to cancer cells. Cell viability assessments resulted in significantly-lower IC<sub>50</sub> dose for ADC (0.47 nM) compared with inhibitor alone treatment (>50 nM) (Fig. 4D). In CAL51 xenografts, ADC or A-419259 treatment resulted in significantly-reduced tumor growth and tumor weights compared with antibody alone or vehicle controls. ADC resulted in significantly-lower tumor weights (14.7  $\pm$  1.4, mg  $\pm$  SEM) compared with vehicle (32.5  $\pm$  2.4 mg,  $P$  < 0.0005) or antibody (39.4  $\pm$  8.8 mg,  $P$  < 0.005) treatments. Tumor weights after inhibitor treatment (19.9  $\pm$  5.0 mg) were significantly-lower than antibody or vehicle controls ( $P$  < 0.05). ADC (7.5 mg/kg, equivalent to 2.66  $\mu$ g A-419259 per mouse) and inhibitor alone (5 mg/kg, equivalent to 0.1 mg A-419259 per mouse) showed similar growth inhibition, although the A-419259 dose coupled with ADC measured only 2.66% of the dose of uncoupled inhibitor (Fig. 4E).

Our findings demonstrate the therapeutic potential of ADC targeting FR $\alpha$  and downstream pathways against breast cancer.

### **MOv18-IgG1 induces tumor cell killing by human immune cells**

We next evaluated the potential of MOv18-IgG1 to activate immune effector cells against cancer cells. In a live-dead cell cytotoxicity imaging assay, human PBMCs pre-labeled with CellTracker Blue dye served as immune effector cells, and CFSE-labelled CAL51 were used as targets. We observed higher rates of dead cells (red fluorescent cells, depicting ethidium homodimer-1 incorporation into dead cells) with MOv18-IgG1 compared with control antibody treatments (Fig. 5A). We quantified tumor cell killing (Antibody-Dependent Cell-mediated Cytotoxicity (ADCC) and Phagocytosis (ADCP)) by MOv18-IgG1 using a flow cytometry-based assay (26). With U937 human monocytes as effector cells (Supplementary Fig. 4A), MOv18-IgG1 mediated killing of FR $\alpha$ -expressing, but not of low FR $\alpha$ -expressing cancer cells by a combination of ADCC and ADCP functions (Fig. 5B; Supplementary Fig. 4C). MOv18-IgG1 induced predominantly ADCP effects (ADCC vs



ADCP ratios: CAL51 = 1.6:1, T47D = 0.4:1, HDQ-P1 = 0.5:1). These effector functions are consistent with previously-reported tumor cell killing engendered by human monocytes and tumor antigen-specific IgG1 antibodies (26, 39).

Breast cancer patients' immune responses may be suppressed, and patient immune profiles may be altered by adjuvant radiotherapy or chemotherapy (40), hence potentially less capable of restricting tumor growth. MOv18-IgG1 could stimulate immune effector cells (PBMC) from healthy volunteers and patients with TNBC (patient characteristics: Supplementary Fig. 4D) to kill CAL51 in an antigen-specific manner (ADCC vs ADCP ratio: healthy volunteers 0.8:1; TNBC patients 0.7:1) (Fig. 5C, Supplementary Fig. 4E and 4F).

These data demonstrate the ability of an anti-FR $\alpha$  antibody to activate patient immune effector cells to induce breast cancer cell death *in vitro*.

### **Anti-FR $\alpha$ antibody treatment restricts the growth of two orthotopic TNBC human xenograft tumors *in vivo***

We examined the potential Fc-mediated anti-tumor effects of MOv18-IgG1 *in vivo*. We employed an orthotopic mammary fat pad-established human TNBC xenograft in immunodeficient mice. This model features impairments in B, T and natural killer (NK) cell development and functions, and lack MHC class I/II expression, designed to minimize the xenogeneic graft-versus-host disease. The model allows introduction of human immune cells to serve as human FcR-expressing effector cells (41), therefore, treatments followed introduction of immune effector cells (freshly-isolated human PBL).

Immunohistochemical evaluations of established xenografts confirmed *in situ* FR $\alpha$  expression in mammary orthotopically-formed CAL51 tumors (Fig. 6A). Tumors from mice given PBL showed human immune cell (CD45<sup>+</sup>) infiltration in immunohistochemical evaluations (Fig. 6B), confirmed by flow cytometric assessments of human CD45<sup>+</sup> cells extracted from xenografts. MOv18-IgG1-treated animals at either 5 or 10 mg/kg dosages showed significantly reduced tumor growth and resected tumor weights (Fig. 6C) compared with controls. Average tumor weight was  $90 \pm 20$  (mg  $\pm$  SEM) for the 5 mg/kg group and  $60 \pm 10$  mg for the 10 mg/kg group, compared to control mice given PBS ( $150 \pm 20$  mg,  $P < 0.05$ ), PBL-alone ( $120 \pm 10$  mg,  $P < 0.005$ ) or antibody-alone ( $170 \pm 20$  mg,  $P < 0.005$ ).

We investigated whether the antibody treatment could also restrict TNBC PDTX growth. Immunohistochemical evaluations revealed that 11 of 26 (46.2%) PDTX tumors were FR $\alpha$



positive (Fig. 6D). We examined the efficacy of MOv18-IgG1 in the fast-growing WHIM02 PDX model that features high (100% of cells) membrane FR $\alpha$  expression. All WHIM02 tumors injected with PBL showed immune cell infiltration in tumor stroma irrespective of treatment (Fig. 6E). Average tumor weight was  $350 \pm 40$  mg in PBL-alone group compared to  $100 \pm 20$  mg with MOv18-IgG1 treatment (71% lower average tumor weight,  $P < 0.0005$ ), suggesting significantly-reduced growth associated with antibody treatment. Although the great proportion of infiltrating CD45<sup>+</sup> human cells were T cells (Supplementary Fig. 5), we measured a modest increase in tumor-infiltrating macrophages ( $3.8 \pm 0.9\%$  with MOv18-IgG1, compared to  $1.2 \pm 0.5\%$  PBS,  $P < 0.05$ ) and NK cells ( $5.7 \pm 1.5\%$  with MOv18-IgG1, compared to  $1.7 \pm 0.5\%$  PBS,  $P < 0.05$ ) (Fig. 6G) associated with antibody treatment. This is consistent with the immune activation seen *in vitro*.

Our results therefore demonstrate significant tumor growth restriction associated with anti-FR $\alpha$  antibody in both orthotopic TNBC line and PDX models. These effects may at least partly be attributed to a responsive, antibody-dependent, immune effector cell mechanism, a notion supported by MOv18-IgG1-mediated tumor/immune cell interactions and ADCC/ADCP functions against cancer cells.

## Discussion

Cytotoxic chemotherapy remains the only systemic treatment modality for patients with TNBC, partly because tumor-associated molecules amenable to targeted therapies including antibodies need to be identified and validated. Our genomic and immunohistochemical evaluations demonstrate that a significant population of TNBCs, including viable post-neoadjuvant chemotherapy residual disease, are likely to overexpress the tumor-associated antigen FR $\alpha$ , and that this antigen participates in the biological functions of breast cancer cells. We show that monoclonal antibody approaches recognizing FR $\alpha$  can offer treatment strategies against TNBC, such as through the design of an antibody conjugate to specifically deliver a signaling blocking agent (Fig. 4).

Avoiding immune destruction is considered a hallmark of cancer (42), yet recent breakthroughs demonstrate that the immune system can play important roles in controlling malignant disease, and that antibodies can provide a means by which immune cells could be directed against cancer. For example, treatment with anti-PD-1 inhibitor antibody pembrolizumab could confer clinical responses in 18.5% of patients with advanced TNBC pre-screened for expression of the ligand PD-L1 in tumors [NCT01848834] (43). Antibodies such as our anti-FR $\alpha$  clone, can also engender immune-mediated cancer cell killing via engagement of Fc receptors-expressing effector cells (monocytes, macrophages, NK cells). We exemplify these properties using patient immune effector cells *in vitro* and in orthotopic and patient-derived TNBC models *in vivo* (Fig. 5-6).

Design of anti-tumor antibodies requires selection of a target, ideally one overexpressed by cancer cells and possibly associated with engendering biological advantages to cancer. This may permit selective recognition of more aggressive cancer cells and may facilitate their destruction by targeted treatments, or engagement and activation of effector cells in the immune stroma. We provide evidence to support further evaluation of FR $\alpha$  as a promising target for treatment of a subset of breast cancers. We report that high FR $\alpha$  expression and dysregulated folate metabolic pathway may be associated with basal-like/TNBC phenotype. Importantly, FR $\alpha$  is expressed in high-risk high-grade disease and in post-neoadjuvant chemotherapy residual tumors, themselves associated with high metastatic relapse. Targeted therapies centered on FR $\alpha$  may also benefit from reported low and restricted FR $\alpha$  expression to a small subset of non-malignant tissues (44). We demonstrate that FR $\alpha$  expression confers proliferative and clonogenic advantages to tumor cells and contributes to pathway activation of Src-family non-receptor tyrosine kinases. FR $\alpha$  expression has been shown to associate with STAT3/JAK signaling before

(37), and, here in this report, to contribute to cancer cell signaling through the Src/ERK pathway. These insights point to new opportunities for targeting FR $\alpha$  and for disrupting its associated signaling cascades using a specific inhibitor (Fig. 3-4).

We demonstrate that FR $\alpha$ -expressing cells can be subjected to human volunteer and TNBC patient-derived immune cell-mediated killing with MOv18-IgG1. These effects were not seen against low FR $\alpha$ -expressing cells, suggesting potentially low or no on-target/off-tumor toxic effects. Lowly-expressing normal tissues, mostly rely on other routes of folate uptake, namely the reduced folate carrier or proton-coupled folate transporter. The ability of this antibody to induce ADCP/ADCC against cancer cells in an antigen-dependent manner supports continued study of this and potentially other antibody strategies as passive immunotherapies for breast tumors. Our investigations of therapeutic efficacy in orthotopic xenografts showed significant reduction in tumor growth in both TNBC line and PDX tumors. Additionally, PDX studies revealed an increase in tumor-infiltrating macrophages and NK cells associated with anti-FR $\alpha$  antibody treatment. This may suggest that targeted therapy with antibodies could present an opportunity to influence the immune stroma, enhance cancer cell recognition by effector cells and ultimately activate these cells to control tumor growth (45, 46). Our findings point to a functionally-active antibody able to prime an anti-tumor immune response that is potentially relevant and translatable to the human cancer setting.

Despite their aggressive clinical behavior, TNBCs tend to initially respond better to neoadjuvant chemotherapy compared with other breast cancer types (47). Furthermore, chemotherapeutic agents may have immunomodulatory activity within the tumor microenvironment, supporting the presence of tumor-infiltrating lymphocytes (48). However, five-year survival rates remain significantly worse in TNBC than in non-TNBC patients, likely driven by chemotherapy-resistant cells, possibly residing in micro-metastatic sites that subsequently lead to lethal clinical recurrence (35). We detected FR $\alpha$ -positive tumors in post-neoadjuvant treated residual TNBCs. As residual TNBCs that contain low densities of tumor-infiltrating immune cells after neoadjuvant chemotherapy have a higher risk of relapse (49), FR $\alpha$ -targeting antibody may present a potential strategy to retain or recruit immune effector cells in tumor stroma.

Past and ongoing clinical evaluations of FR $\alpha$ -targeted therapies and monoclonal antibodies, provide ground for cautious optimism. Vintafolide (MK-8109/EC145) is being evaluated in a phase III trial for ovarian cancer [NCT01170650] and a phase IIb trial for non-small cell lung cancer [NCT01577654]. A phase II trial of vintafolide in FR $\alpha$ -positive

TNBC is expected. The concept of using FR $\alpha$ -specific anti-folate drugs is evaluated in an early phase I trial of the first-in-class thymidylate synthase inhibitor, ONX-0801, in solid tumors [NCT02360345] (19). Furthermore, with the same specificity for an epitope of FR $\alpha$ , the MOv18-IgE isotype is being evaluated in a first-in-class clinical trial for ovarian cancer [NCT02546921] (50). Clinical experience with these agents points to a need for improved patient selection and for elucidating mechanisms of action. In breast cancer, FR $\alpha$  expression levels could be used for patient stratification. Perhaps tumor infiltration of key effector cells that may be activated against highly-aggressive or chemotherapy-resistant cancer cells, could also be employed to monitor treatment responses or to select patients more likely to benefit.

Collectively, our findings support FR $\alpha$  expression at the transcriptomic and protein levels, and cell surface expression in a proportion of basal-like and TNBC subtypes, including in neoadjuvant chemotherapy-resistant tumors. We report associations of higher expression with worse clinical outcomes, and evidence for functional significance in breast cancer cell biology. We demonstrate the potential tumor-restricting effects of anti-FR $\alpha$  MOv18-IgG1; by potentiating immune effector cell activation and cancer cell-neutralizing functions *in vitro* and by restricting tumor growth in orthotopic TNBC line and PDX models *in vivo*; by an ADC design approach of the anti-FR $\alpha$  antibody coupled with a Src-family kinase inhibitor *in vitro* and *in vivo*. Our findings point to FR $\alpha$  as a promising antigen for different antibody therapy approaches and may provide the basis for further translational investigations, effective patient stratification and personalized therapies, especially for patients who do not adequately benefit from currently-available treatments.

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## Figure legends

### Figure 1. Basal-like/TNBC is associated with upregulated FR $\alpha$ gene expression

Gene expression in METABRIC, TCGA and KCL datasets for *FOLR1*, *MTHFR*, *TYMS* and *FOLH1*. **(A)** Cohorts were divided into TNBC and non-TNBC based on IHC-defined receptor status. **(B)** Cohorts above were stratified according to PAM50 classification (Basal-like (Basal), HER2, luminal A (L.A), luminal B (L.B) and normal-like (N.L.)). Median-centered gene expression log2 values are shown. Numbers of patients per group is indicated below the graphs in the first column. *P*-values were determined using the Wilcoxon rank-sum test. **(C)** Relationship between *FOLR1* and *FOLH1* in the KCL dataset. **(D)** Association of FR $\alpha$  expression (upper quartile) with ten-year overall survival. **(i)** Kaplan-Meier curves in 1,402 breast cancer samples, and **(ii)** TNBC subset with 47 samples. The number of patients per group is indicated below. Significant *P*-values are indicated with an asterisk, where \* *P* < 0.05; \*\* *P* < 0.005; \*\*\* *P* < 0.0005.

### Figure 2. FR $\alpha$ expression is associated with high grade breast cancer and basal-like/TNBC, and is found in chemotherapy-resistant residual TNBC tumors.

**(A)** IHC staining for FR $\alpha$  membrane expression in KCL TMA. Representative images showing restricted expression in normal human kidney and lung sections, **(i)** a case with no FR $\alpha$  expression and negative staining, **(ii-v)** positive cell surface cancer cell FR $\alpha$  staining, score: 10% to 100%. Data were classified based on tumor grade and IHC- or PAM50-defined receptor status. Each group with the highest population of positive cancer cell surface staining were displayed in pie chart subdivided into three sectors (low, medium and high score based on % membrane FR $\alpha$  staining). **(B)** Microarray-based FR $\alpha$  mRNA expression were compared to membrane FR $\alpha$  staining positivity tested by IHC staining. **(C)** IHC staining for membrane FR $\alpha$  expression in KCL post-neoadjuvant chemotherapy residual TNBC tumor TMA. **(i-iv)** Representative images showing FR $\alpha$  staining, score: 0% to 100%. 11 out of 18 samples were found to be positive for FR $\alpha$  expression. Pie chart subdivided the samples into three sectors (low, medium and high score based on % of

cells positive for membrane FR $\alpha$  staining). The proportion of patients per group is indicated below. Significant *P*-value is indicated with an asterisk where \*\*\* *P* < 0.0005.

### **Figure 3. Surface FR $\alpha$ protein levels in breast cancer cell lines and RNA interference of FR $\alpha$ leads to reduction in cellular activities**

(A) Surface FR $\alpha$  expression of twenty-two breast cancer cell lines were evaluated by flow cytometry using MOv18-IgG1. Histopathological subtype of each cell line was listed on Supplementary Fig. 2A. Cell lines with high surface FR $\alpha$  level that were ultimately selected for further analysis are highlighted (CAL51, red; T47D, blue; HDQ-P1, pink, also see representative histograms of flow cytometric evaluations of FR $\alpha$  expression). The evaluations also included the widely studied breast cancer surface receptors EGFR and HER2 as internal controls (Supplementary Fig. 2B and 2C). (B) FR $\alpha$  mRNA expression data of the cell lines were extracted from CCLE database. Analyses showed a positive correlation ( $r = 0.5784$ ) between protein and mRNA levels of expression (Spearman's Rank coefficient analysis, *P* < 0.01). (C) Significant restrictions in cellular growth after 96 hr of siRNA-mediated silencing of FR $\alpha$ , and reduction in colony density over a ten-day period, were shown only in the FR $\alpha$ -positive cell lines. (D) FR $\alpha$  expression in CAL51 cells transduced with non-targeting shRNA (shNT) and FR $\alpha$ -targeting shRNA (shFR $\alpha$ ) were represented as MFI based on MOv18-IgG1 staining. CAL51 demonstrated growth restriction, visible reduction in colony density, and decreased ERK activity with stable FR $\alpha$  knockdown. (E) Parental CAL51 were treated with raltitrexed in both normal and folate-free conditions. Cells with FR $\alpha$  knockdown were less sensitive to the treatment in both conditions. The data represent the mean  $\pm$  SEM values of at least three independent experiments. \* *P* < 0.05; \*\* *P* < 0.005; \*\*\* *P* < 0.0005, by two-tailed unpaired t-test.

### **Figure 4. FR $\alpha$ modulates phosphorylation of targetable signaling molecules and antibody-drug conjugate inhibition of tumor growth.**

(A) Images from Proteome Profiler Human Phospho-Kinase Array (decrease in phosphorylation marked in red; increase in phosphorylation marked in green). Each kinase is spotted in duplicate. Loading reference points at lower exposure for each membrane are shown in Supplementary Fig. 3B. Pixel densitometry analysis was expressed as fold change comparing the shFR $\alpha$  sample to corresponding shNT sample. (B) Cells were treated with broad spectrum Src-family kinase inhibitor A-419259 to assess the dose dependent inhibition of cellular growth. Half-maximal inhibitory concentration (IC<sub>50</sub>) doses

were determined with MTT assay following 96 hr incubation. CAL51 incubated with A-419259 had shown visible reduction in colony density over a three-week period, where the inhibitor was refreshed weekly, and decreased ERK activity after 4 hr of drug treatment. **(C)** A model depicting FR $\alpha$ -mediated regulation of cancer signaling and as folate transporter for cell growth and survival. **(D)** Viability assessment of FR $\alpha$ -targeting MOv18-IgG1-coupled inhibitor ADC-treated CAL51 cells compared with MOv18-IgG1- and A-419259- treated cells. Data are means  $\pm$  SEM from N = 3 independent experiments. **(E)** Growth curves and weight measurements of resected CAL51 tumors (N=10 mice per treatment group) treated with a single-dose of ADC (7.5mg/kg), MOv18-IgG1 (7.5mg/kg), A-419259 (5 mg/kg) or PBS. \*  $P < 0.05$ ; \*\*  $P < 0.005$ ; \*\*\*  $P < 0.0005$ , by two-tailed unpaired t-test.

### Figure 5. MOv18-IgG1 antibody induces immunotherapeutic tumor cell killing

**(A)** Fluorescent images of the live cell cytotoxicity assay. Live CFSE-labelled CAL51 tumor cells (green) were incubated for 24 hr with MOv18-IgG1 or isotype antibody and PBMC (stained with CellTracker Blue dye). Incorporation of ethidium homodimer-1 is depicted as red fluorescence into damaged cells, was observed. **(B)** FFPE cell pellets of six breast cancer cell lines (CAL51, T47D, HDQ-P1, SKBR3, MDA-MB-231 and HCC1428) were cut and stained for evaluation of FR $\alpha$  expression. Breast cancer cells were treated with 5  $\mu$ g/ml MOv18-IgG1, or with isotype-matched control antibody. Human U937 monocytic cells were added to the tumor cells and incubated for 3 hr at 37°C followed by the flow cytometry-based tumor cell killing assay to determine the levels of ADCC and ADCP of cancer cells (N = 3). **(C)** Healthy volunteer PBMCs (N = 8), and TNBC patient PBMCs (N = 9) were also used, results were illustrated as total % tumor cell killing and as separated ADCC (black) and ADCP (grey). MOv18-IgG1 appeared to induce ADCP-biased anti-tumor effects. All the data represent the mean  $\pm$  SEM values of three independent experiments. \*  $P < 0.05$ ; \*\*  $P < 0.005$ ; \*\*\*  $P < 0.0005$ , by two-tailed unpaired t-test.

### Figure 6. Restriction of orthotopic tumor growth *in vivo*

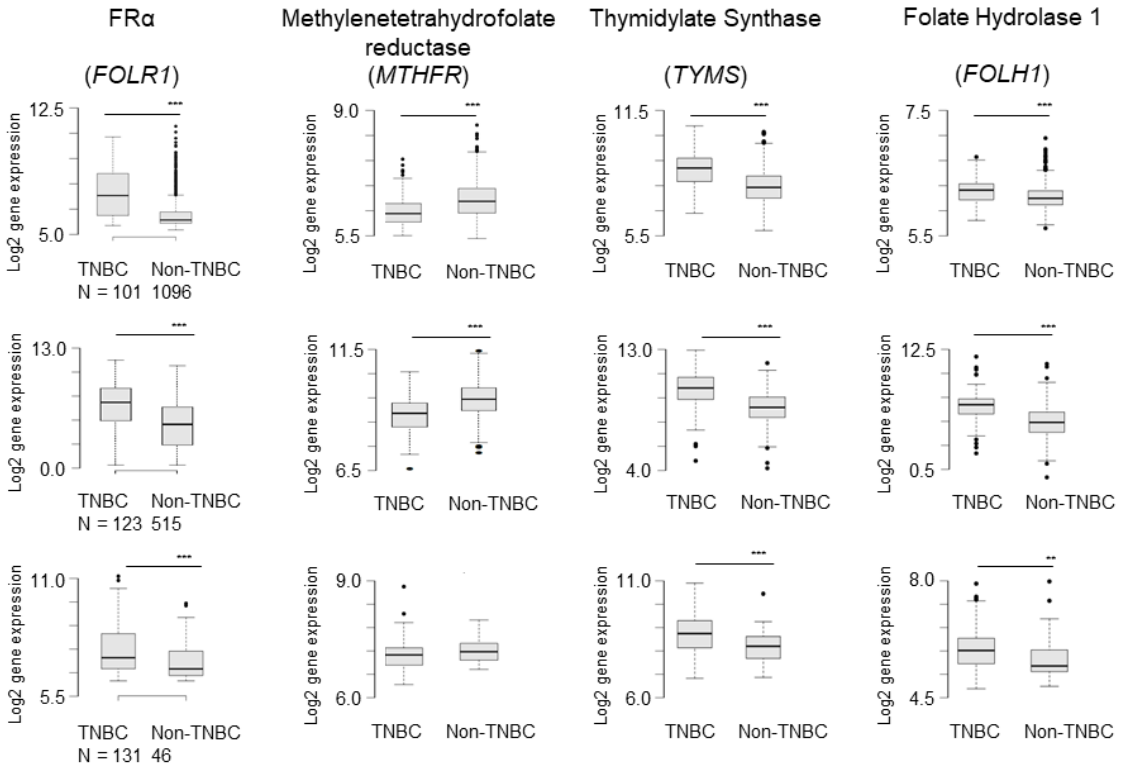
**(A)** IHC evaluation of FR $\alpha$  expression in paraffin-embedded CAL51 xenograft tumor specimens. **(B)** Tumor engraftment of human immune cells were confirmed by anti-human CD45 IHC staining in tissue sections. **(C)** Growth curves and weight measurements of resected CAL51 tumors of the partly immuno-humanized mice treated with 5 or 10 mg/kg MOv18-IgG1 antibodies. **(D)** IHC evaluation of FR $\alpha$  expression in a TMA of 26 PDTX

models. Representative images showing no FR $\alpha$  expression in KCL005 and 100% positive FR $\alpha$  staining in WHIM02, with 43.2% of the TNBC PDX models (N = 26) shown to be positive of FR $\alpha$  expression. **(E)** Tumor engraftment of human immune cells was confirmed by anti-human CD45 IHC staining. **(F)** Growth curves and weight measurements of resected WHIM02 PDX tumors of the partly-immuno-humanized mice treated with 10 mg/kg MOv18-IgG1. **(G)** Flow cytometric analyses demonstrating engraftment of CD45+ human immune cells in the WHIM02 PDX model, and infiltrating immune cell populations of potential effector cells (human macrophages and NK cells). Data are means  $\pm$  SEM \*  $P < 0.05$ ; \*\*  $P < 0.005$ ; \*\*\*  $P < 0.0005$ , by two-tailed unpaired t-test.

Figure 1

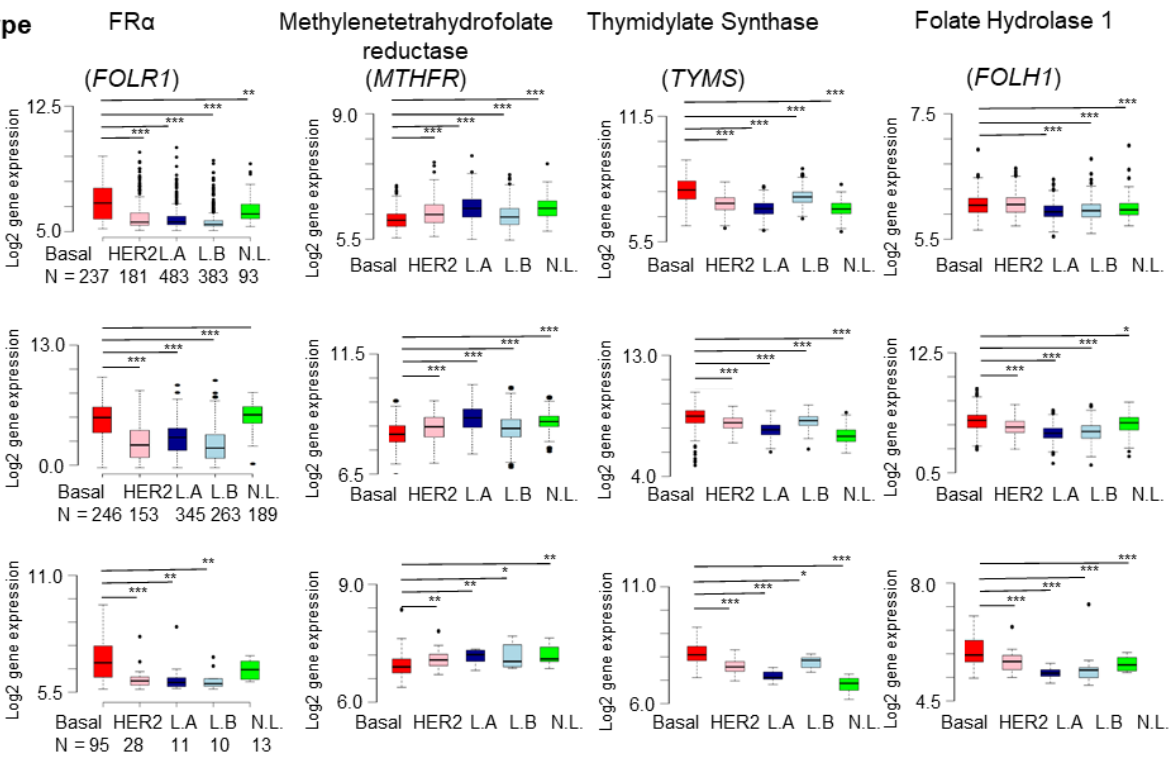
(A) IHC subtype

METABRIC dataset

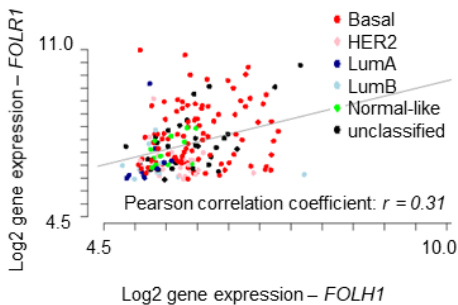


(B) PAM50 subtype

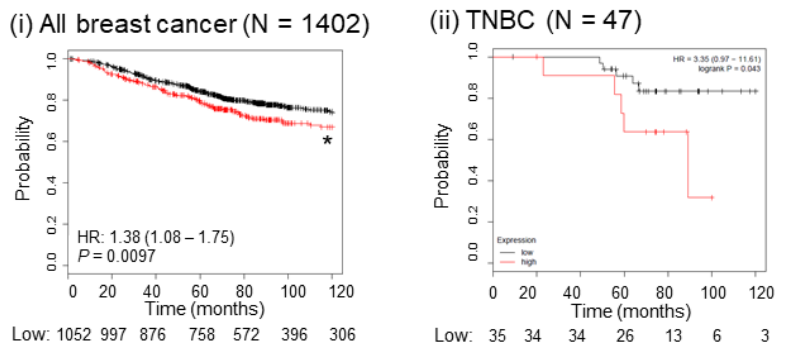
METABRIC dataset



(C) FRα expression versus Folate Hydrolase 1 expression (KCL dataset N = 177)



(D) Overall survival based on FRα expression (upper quartile):

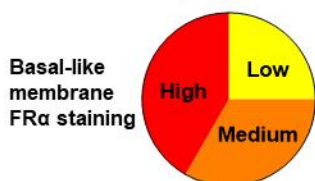
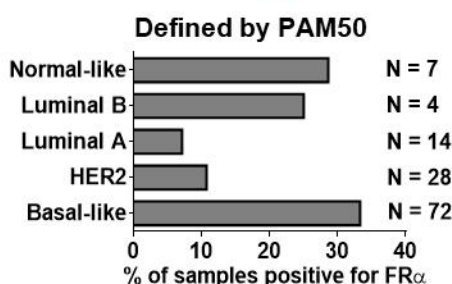
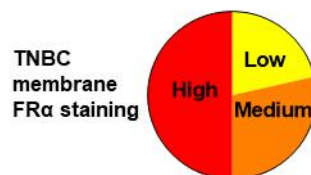
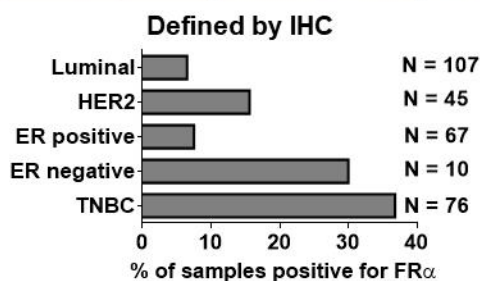
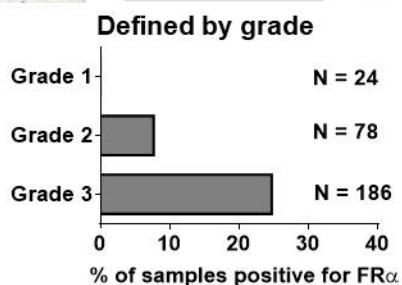
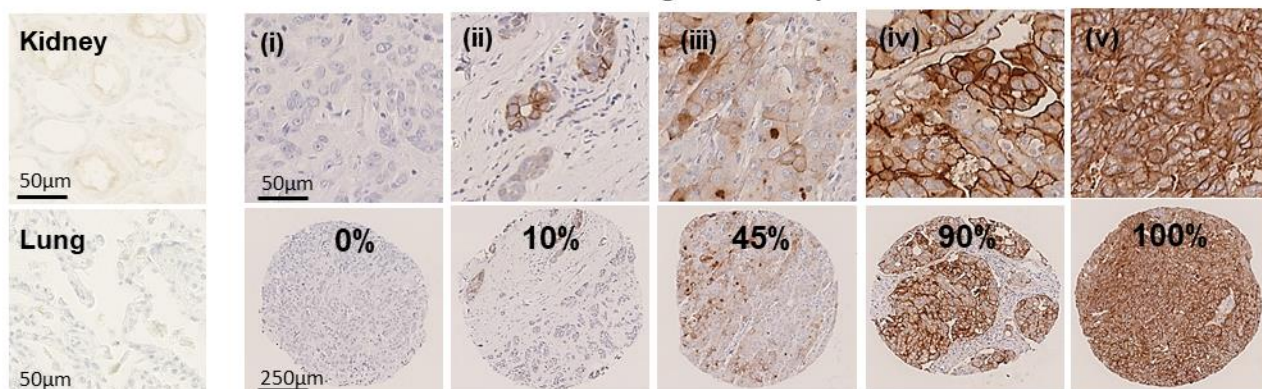




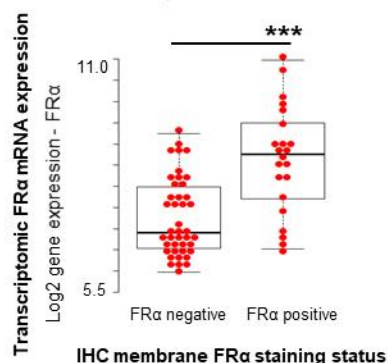
**Figure 2**

**(A) King's College London breast cancer TMA**

**FR $\alpha$  membrane staining: % of FR $\alpha$  positive tumor cells**

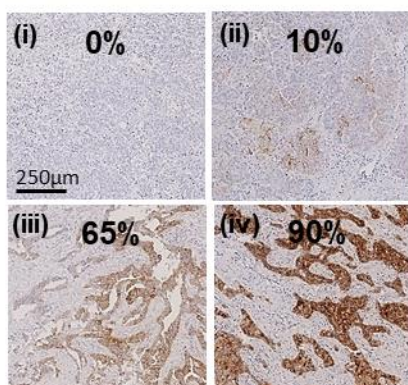


**(B) Microarray-based FR $\alpha$  expression versus membrane FR $\alpha$  staining positivity (KCL basal dataset)**



**(C) King's College London TNBC residual tumor TMA**

**FR $\alpha$  membrane staining: % of FR $\alpha$  positive tumor cells**



**TNBC residual tumor TMA**

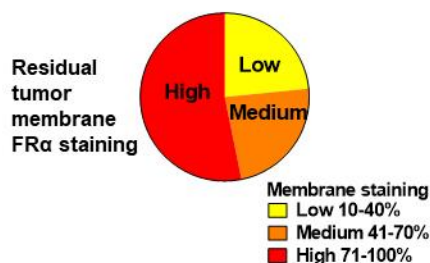
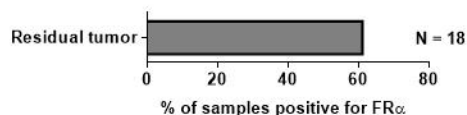




Figure 3

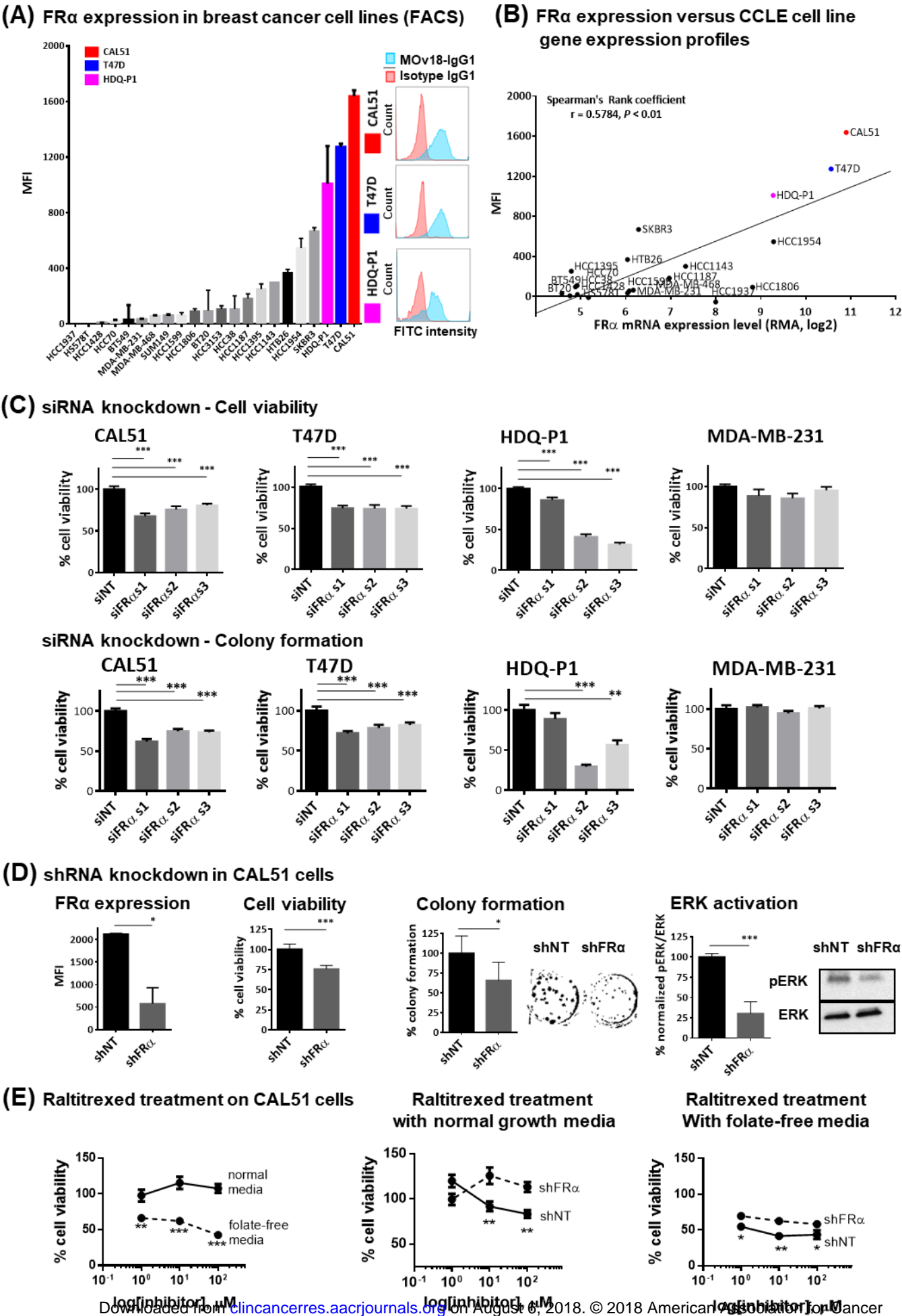
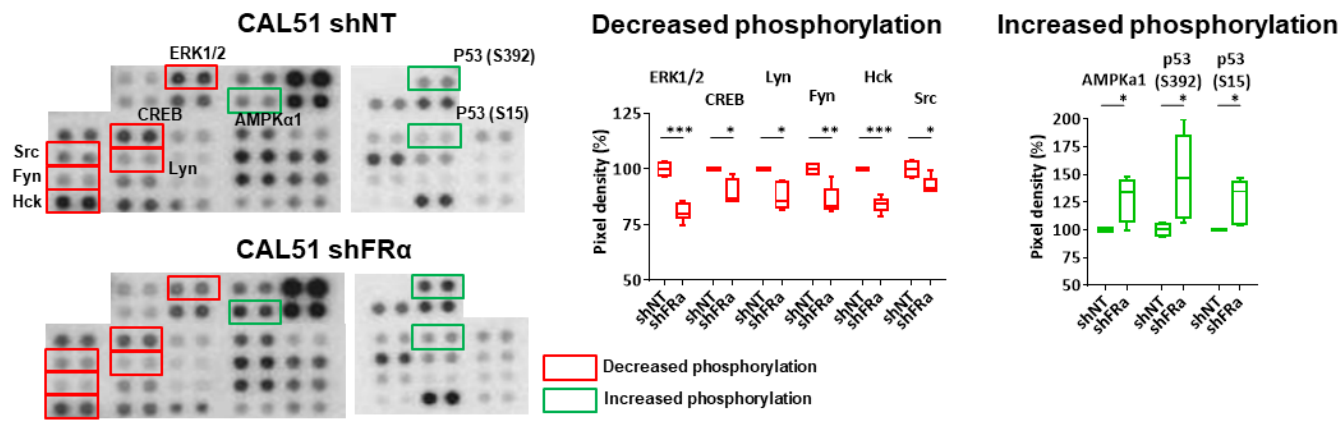
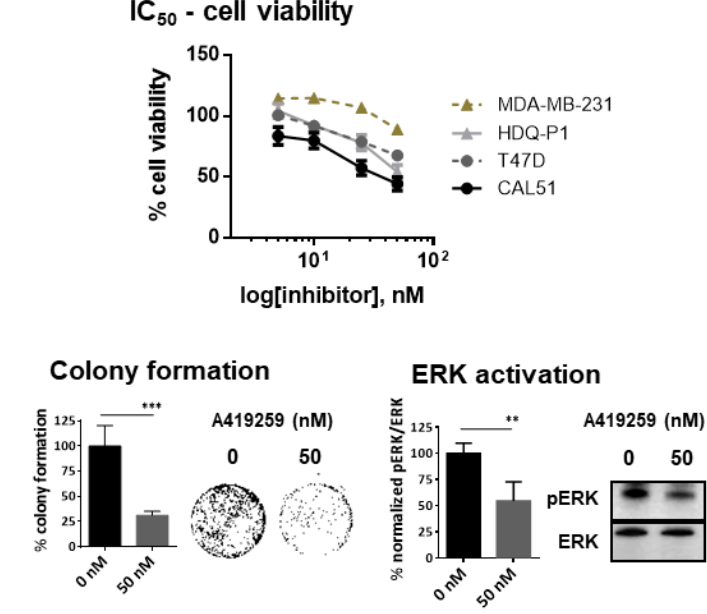


Figure 4

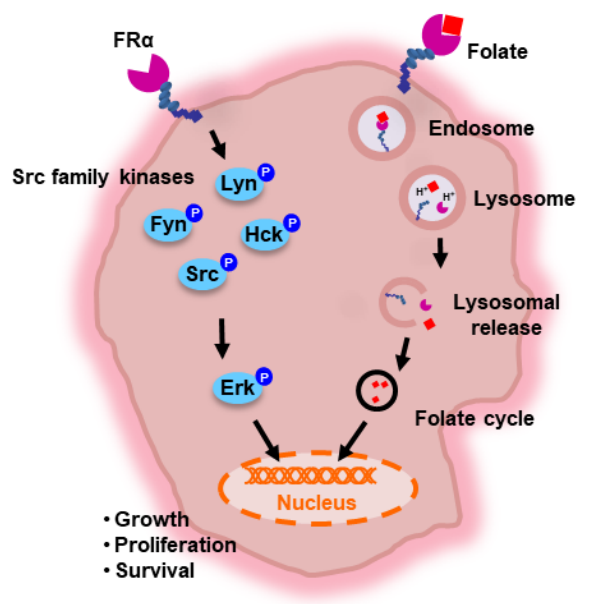
(A) Human Phospho-Kinase Array



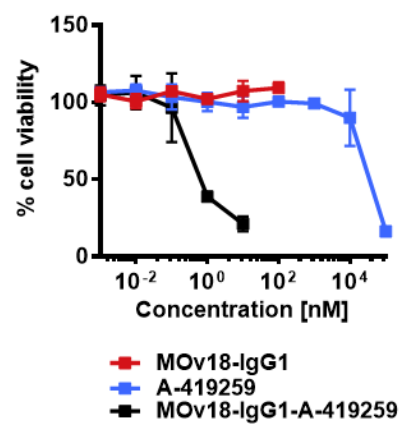
(B) Src family kinase inhibitor A-419259



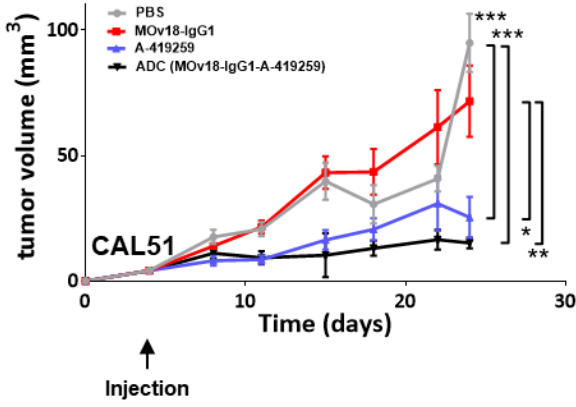
(C) FRα-mediated cancer growth



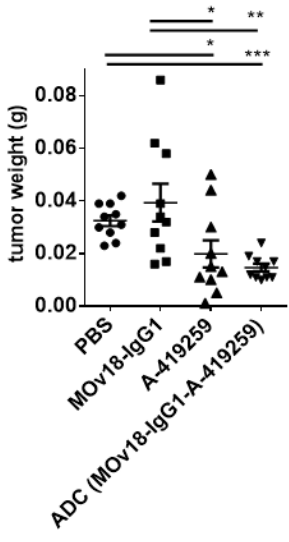
(D) Antibody-drug conjugate *in vitro* cell viability



(E) Antibody-drug conjugate *in vivo* growth curve

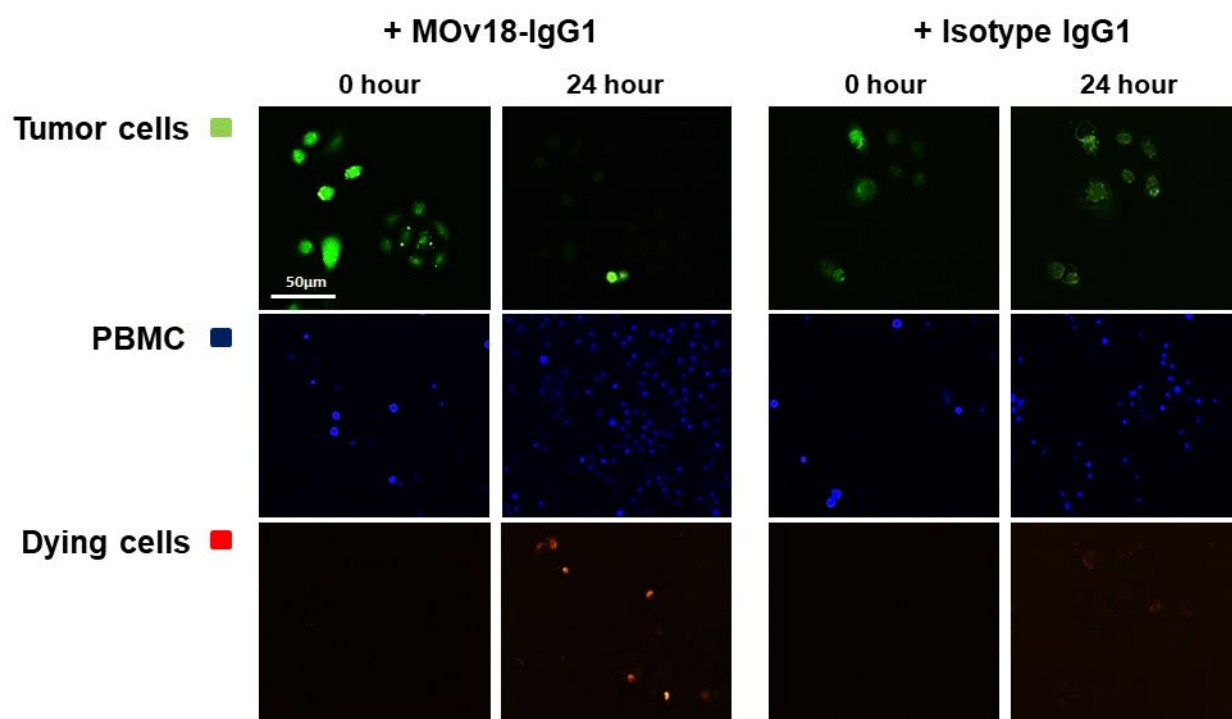


Resected tumor weight

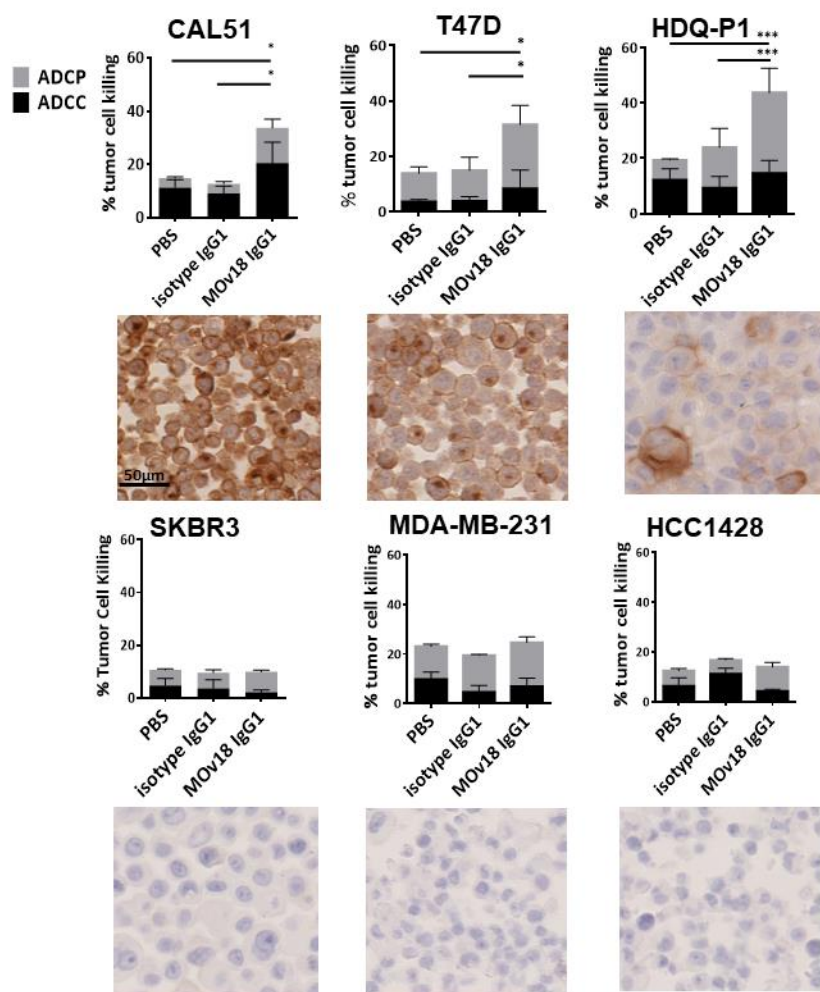


**Figure 5**

**(A)** Immunofluorescence microscopy – interaction between cancer cells and immune cells



**(B)** FR $\alpha$ -dependent immune cell killing – U937 monocytic cells



**(C)** Human blood sample

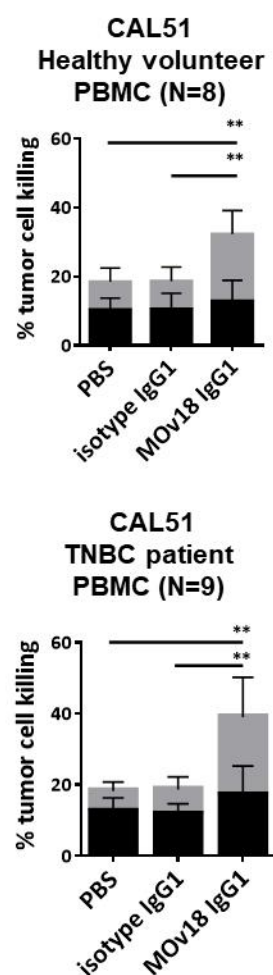
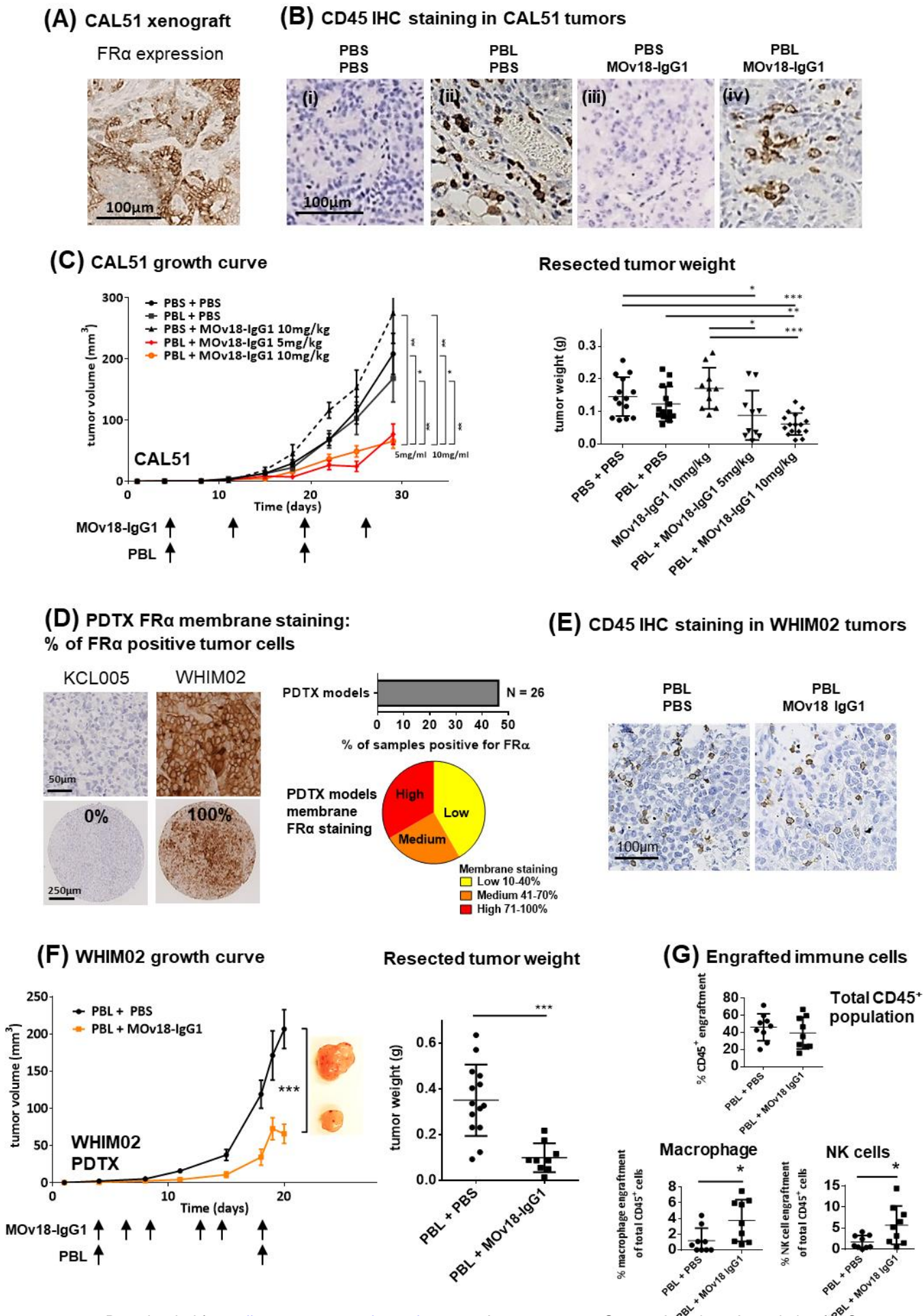




Figure 6



# Clinical Cancer Research

## Anti-Folate Receptor alpha-directed Antibody Therapies Restrict the Growth of Triple Negative Breast Cancer

Anthony Cheung, James W. Opzoomer, Kristina M Ilieva, et al.

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